GENOME TO PHENOME: INTEGROMIC APPROACHES TO DEFINE NETWORKS OF HOST-PATHOGEN INTERACTIONS AND VACCINE BIOMARKER DISCOVERY

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ABSTRACT

The rapid development of genomics, proteomics and high-throughput computing approaches to study diseases is now allowing us to apply an integrative systems biology work frame for drug and biomarker development. Understanding the symbiosis of pathogen and host at the molecular level requires a comprehension of the molecular/cellular events by which the pathogens can induce alterations in the host in order to invade, proliferate and overtake the host. Our research focuses on defining the molecular events/network systems involved in the host-pathogen interactions to identify biomarkers and therapeutic targets for pathogenic agents.

Host-pathogen interactions and biomarkers discovery:

To understand the complex interaction between various biological pathogens and the host, we have applied global gene profiling to determine basis of infectious or biothreat-induced diseases and to identify host defense strategies and the mechanisms by which they are regulated. Although gene response profiles show unique signatures quite rapidly after exposure, they also have the potential to reveal phases of progression of illness to a) provide stage-specific diagnosis and b) identify potential molecular targets for stage-appropriate therapeutic interventions for intractable illnesses induced by unconventional pathogenic agents.

We are using human peripheral mononuclear cells (PBMC) and other tissues to study whole genome regulation in response to various pathogenic agents and to identify diagnostic biomarkers for these pathogens. We apply various algorithms to extract features and signatures that can be used for point-of-care diagnosis of various infections. We have also developed two software packages that will enhance the analysis of high throughput omics experiments with high yielding results.

We have studied the kinetics of host responses to various groups of pathogens and identified key networks/nodes regulated by each of these pathogens. These studies were complemented by other assays and clinical manifestation using in vivo animal models, such as WBC, blood pressure, body temperature…etc.

Integrating genomic/proteomic studies with clinical findings to understand the course of SEB-induced shock:

We have used piglets as an animal model for SEB induced shock and carried out genomic and proteomic analyses. Piglets were challenged with SEB at various time points (up to 96hrs) and blood/tissue samples were analyzed to establish the dynamics of host responses to SEB.

Our results show that SEB stimulates a Th1 and cytokine responses with an upregulation of TNF-α, IL-1A, IL-6, IFN-γ, and several interleukins, even by 2h. HIF-1α was also upregulated by 2h and continued to rise through 48 h. Pathways and networks controlled by HIF-1αshowed their major increases by 24h including the production of nitric oxide by 24 h in the plasma. The kinetics of the regulation of blood pressure correlated with the expression of genes coding for many regulators such as angiotensin, angiotensin converting enzyme, vasopressin receptors and many other mediators that might appear to logically result in vascular stabilization.

Gene ontological classification were carried out using FATIGO^{+}, GeneCite, PAINT and the Ingenuity pathway analysis (IPA) to identify pathways and transcription factor networks regulated by SEB. These targets are all interconnected and are known to be regulated by TNF-α. This network is also found to be interacting with 3 other network nodes that were regulated by SEB (Jun, Caspase 3 and CREB1). *in silico* analyses were carried out to construct qualitative models based on the simple protein/protein-complex reactions and transcription factor activation and were experimentally validated in vitro to refine our findings.

The systems biology approach used in this study involves integration of multiple data types including clinical findings, various -omic approaches, information relating to individual/ multiple tissues affected from the onset of the exposures. Assembling this broad network of information has provided a framework by which we can begin to identify markers of illness progression and correlate with appropriate therapeutic strategies as the illness proceeds. Our aim is to eventually identify markers that will predict the eventual course of impending illness. One would like to be able to differentiate, at very early time periods, the eventual onset of a

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common, minor malaise from what will become a serious illness, as well as the nature of that serious illness. Mapping the regulatory networks involved in the progression of infection or disease offers important insights that have the potential to lead to a broad understanding of immune modulation and shift of paradigm from the standard "one-bug-one drug" to overarching stimulation/inhibition of critical host responses.

1. INTRODUCTION

There is an urgent need to assess exposure and infection of individuals by biological threat agents (BTA) well in advance of onset of illness or at various stages post-exposure. Direct pathogen identification can be elusive since many pathogens sequester in tissues initially. Direct pathogen methods include the classical culture methods, immunoassay, and gene amplification of the pathogen. Although these methods are being improved for incredibly greater sensitivity [1-3], the efficiency of any diagnostic approach for direct pathogen assessment depends upon the presence of agent in a small specimen matrix. By the time detectable levels of pathogen are reached, it is frequently too late to halt the progression of the intractable illness [4, 5].

Host responses can occur rapidly after exposure to specific pathogenic agents could provide the needed information for defense against the biothreat agents at time periods when clinical signs might include general malaise or flu-like symptoms that would not differentiate among diverse pathogenic agents.

1.1 Host-pathogen interactions and biomarkers discovery:

Correlation of the course of the infection and the disease progression with molecular responses provides opportunities to understand pathogenesis resulting from biological agent exposure. Symptoms of exposure to biological toxins such as *Vibrio cholerae* toxin (CT), *Clostridium botulinum* toxin A (BoNT-A), and Staphylococcal enterotoxin B (SEB) include violent reactions within hours of exposure and may lead to death in a few days. In contrast, *Brucella* infection *(B. melitensis* 16M*)* produces late-onset of mild symptoms that persist over a long period of time. Other bacterial pathogens, such as those causing anthrax (*B. anthracis*) or plague (*Yersinia pestis*), induce systemic, flu-like symptoms initially and progress to lethal shock and death days later.

In the case of viral threat agents, Venezuelan equine encephalitis (VEE) initially causes serious illness, associated with severe malaise and an extended recovery time. Dengue (DEN-2) patients usually recover fully within days (< 1% mortality), but some individuals, especially those with prior dengue exposure, may develop dengue hemorrhagic fever (DHF). Therefore, the time when an individual is exposed, the incubation period, and the time of manifestation of the illness are crucial to designing diagnostic and therapeutic strategies.

In the post-genomic period, it is no longer unrealistic to hope that the examination of host responses, by interrogating large numbers of genes, could reveal unique responses to the various BTA. However, there are a number of obstacles yet to be overcome. Different agents may affect different tissues or cells that may not be available for diagnostic purposes. For example, botulinum toxins target neuromuscular synapses and cholera toxin aims for intestinal epithelial cells.

As a first step toward the goal, we focused on gene expression changes in human peripheral blood mononuclear cells (PBMC) since they could be readily obtained from an exposed individual, thus taking advantage of their "reconnaissance" role. We carried out in vitro exposure to each of 8 biological threat or infectious agents. We confirmed the in vitro results by using peripheral blood mononuclear cells (PBMC) from nonhuman primates (NHP) exposed to a bacterial pathogen (*Bacillus anthracis*) or, separately, to a toxin (SEB) at various time points post-exposure to compare findings in vitro to those seen in vivo. We have identified host gene expression patterns that can discriminate exposure to various BTA, even at early time periods when flu-like symptoms occur.

1.2 Integrating genomic/proteomic studies with clinical findings to understand the course of SEB-induced shock:

We used piglet models to study the pathogenesis of SEB using genomic and proteomic approaches and correlate the finding with the histopathology in exposed animals. Piglets were challenged with

SEB at various time points (up to 96hrs) and blood/tissue samples were analyzed to establish the dynamics of host responses to SEB.

Figure 1 depicts the stages of shock that were correlated with a number of observations including edema, appearance of fibrin clots (increasing severity is illustrated with the increased color intensity of the bar graph). The blood pressure showed a 30% decrease in systolic pressure by 24 h. From 24-60 h, stabilization of blood pressure occurs (gene profiles suggest the pathways involved in this stabilization). With severe edema, capillary pooling, etc, the vascular collapse is observed from 72-96 h. Just below the graph are light blue arrows indicating the time periods at which peripheral blood leukocytes were used for genomic studies and other tests. TEG measurements (red bars) characterize a compilation of clotting factors, platelet function, fibrinolysis, etc to describe clotting efficiency. The increase, through 36h, suggests activation of the clotting cascade by 18h. At 48 h, the decrease observed may relate to leakage of clotting factors or platelets into the interstitial fluids, among other possibilities. At the bottom of the graph are micrographs showing swine mesenteric lymph nodes (normal at the far left). By 48 h, edema was becoming severe and by 72 h, hemorrhage into the tissues was observed.

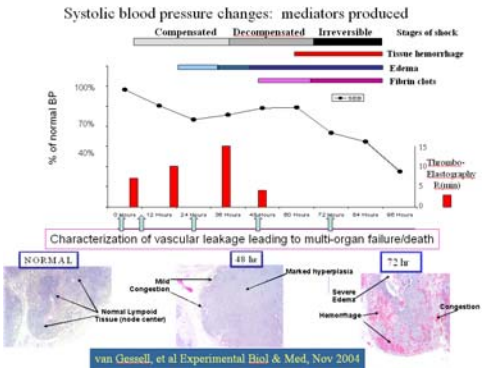

FIGURE 1. Summary of piglet responses to SEB.

Genomic studies were carried out on piglets challenged with SEB at the time periods indicated on Figure 1 and all of the studies were done at 0, 6, 24, 48 and 72 h post exposure. Some studies include 2h or 96 h time periods as well.

Figure 2 shows that the "cytokine storm" in the piglet is reflective of that which has been observed in other studies although in our past NHP exposure studies, frequent measurements such as these were impossible due to the animal expenses and difficulties with handling or anesthesia. In one NHP study, we saw TNF-α in plasma from 1.5-3 h, but no other time periods were available. For the piglet genomic analysis (Fig 2, upper graph), 6h (maroon bar) was the earliest time period examined and IL-18 was the most dramatically upregulated cytokine gene (the corresponding protein protects NK cells). Although small increases were seen at 6 h in TNF-α, IL-1α and IL6, it could be that these were a second wave of modest increases. Those genes were very possibly upregulated at earlier time periods.

We carried out these same studies using spleens from sham/SEB piglets and observed upregulation of many cytokine receptors -and need to characterize that finding more thoroughly. The plasma concentrations of cytokines (using swine antibodies to 9 cytokines, Pierce "Searchlight" dot blot, Elispot assay) were determined starting at 2 h and the most dramatic observation was the rapid, massive increases in INF γ. There appears to be an attempt at homeostasis, as one sees the rapid and large increases in IL-10.

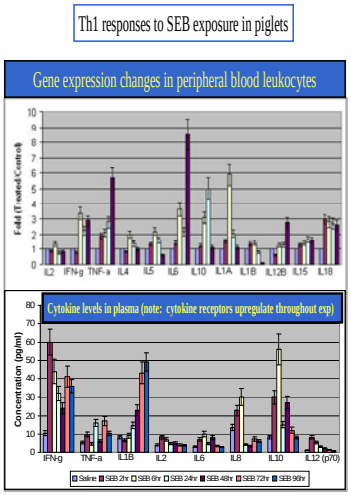

FIGURE 2. SEB stimulates a Th1 response in piglets. The top graph shows the genomic levels of various cytokines using peripheral blood leukocytes upon exposure of piglets to SEB. These studies are compared with animals undergoing sham exposure ("0", 6, 24 (yellow), 48-(light blue), 72 h-(maroon). IL-18 was x 3 upregulated at the first measurement (6h) and it has been shown to protect NK cells (96), as we found in this study. Upregulation of TNF-α, IL-1A, IL-6 are seen a 6h and markedly increase by 24h.

HIF-1α was upregulated by 2h (Figure 3, Graph #1) and continued to rise through 48 h. The pathways that it affects showed their major increases by 24h (light blue bar in graphs #1, 3, 4). Please note that 2-fold increases do not appear dramatic (to the eye!) on the graphs, since some genes upregulate 6-12 fold. We expect that even a 2-fold increase could make

major perturbations in these finely regulated systems. The production of nitric oxide by 24 h offers another possible target for regulation of the eventual outcome. As can be seen from these studies, we need data for more frequent time periods to be able to narrow windows of opportunity. The sudden drop at 72 h may reflect its regulators such as PI-3 kinase, P-38, etc, or simply the glucose transport, which HIF-1 altered by 24 h.

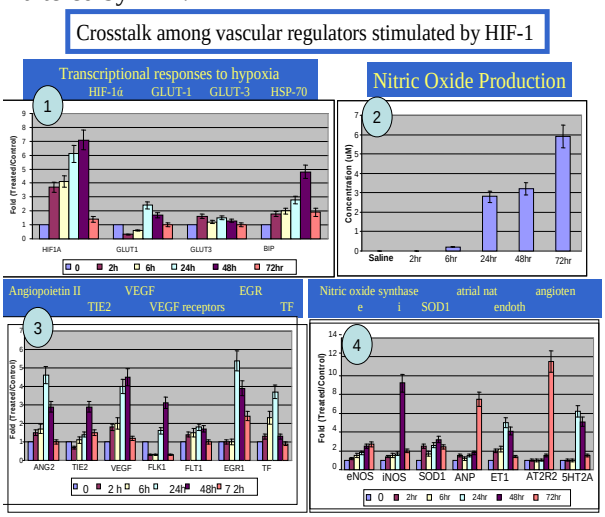

FIGURE 3. HIF-1α and crosstalk among vascular regulators. Graph #1 (Top, left) shows upregulation of HIF-1α at 2 & 6 h, with additional increases at 24, 48 h, but returning to baseline at 72h. HIF-1α has been characterized as stimulating glucose transporters (GLUT), in graph #3 VEGF, Tissue factors (TF); and in graph #4, nitric oxide synthase (NOS), Endothelin (ET1) and other related regulators. Graph 2 shows actual production of Nitric oxide by 24h & 48 h and the amount produced doubled by 72 h.

As we have reported, stabilization of the blood pressure from 24-60 h (Fig. 1) was preceded by increased expression of genes coding for many regulators such as angiotensin, angiotensin converting enzyme, vasopressin receptors and many other mediators that might appear to logically result in vascular stabilization. However, as vascular leakage proceeds (>60 h) that physiologic approach appears to have devastating effects, resulting in hemorrhage into the tissues (Fig. 1).

Genomic studies of primary cultures of human renal proximal tubule endothelial cells (RPTEC) in vitro and in kidneys from SEB challenged piglets (lower corner). In the course of determining SEB effects on RPTEC, we studied phosphorylation cascades and genomic responses to exposures in vitro.

Gene ontological classification were carried out using FATIGO+, GeneCite, PAINT and the Ingenuity pathway analysis (IPA) to identify pathways and transcription factor networks regulated by SEB. Figure 4 depicts one of the networks regulated by SEB showing the up regulated targets in red. These targets are all interconnected and are known to be regulated by TNF-α. This network is also found to be interacting with 3 other network nodes that were regulated by SEB (Jun, Caspase 3 and CREB1). in silico analyses were carried out to construct qualitative models based on the simple protein/protein-complex reactions and transcription factor activation and were experimentally validated in vitro to refine our findings

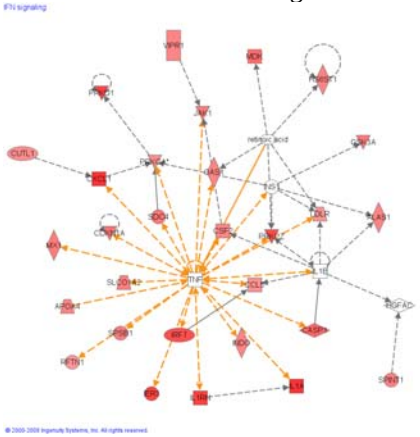

FIGURE 4. Network analysis of genes up regulated by SEB.

1.3 Microarray analysis of host responses to Venezuelan Equine Encephalitis in vitro in PBMC:

We carried out microarray gene expression analysis of PBMC samples exposed to VEE at various time points. We identified genes that exhibited highly statistically significant differential expression in PBMC exposed to VEE at various time points when compared to control untreated cells (Figure 5).

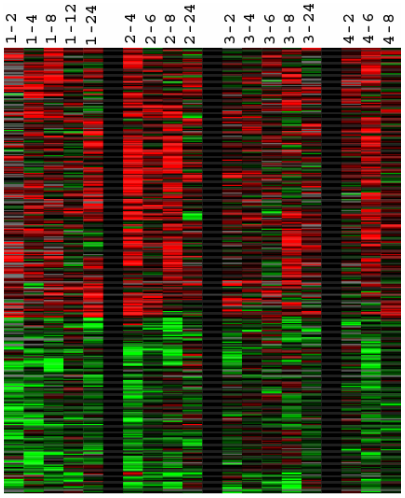

FIGURE 5. Cluster analysis of genes that were differentially expressed in PBMC exposed to VEE at various time points when compared to control untreated cells.

Microarray analysis of host responses to VEE in vivo in NHPs:

We carried out microarray analyses using blood samples obtained from 12 control (unexposed) NHPs along with 8 samples from VEE-exposed NHPs. Although no signs of illness were apparent by day 3, gene expression patterns identified these NHP as having mild exposure to the virus.
Genes that were significantly altered by VEE were clustered to show their expression profiles. These genes were consistently regulated by VEE (figure 6).

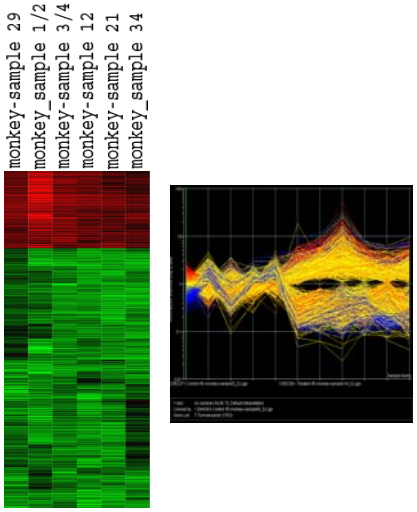

FIGURE 6. Cluster view and expression profiles of genes that were differentially expressed between NHPs exposed to VEE and control NHPs.

We carried out data mining and functional classification using our in-house developed tools for pathway and literature mining showed that some of the genes that were highly up-regulated by VEE were involved in apoptotic pathways and cell cycle (Figure 7).

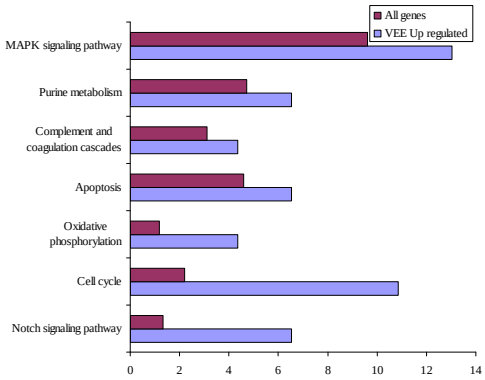

FIGURE 7. Functional analysis of genes that were highly up regulated by VEE in the treated NHPs.

1.4 Use of predictive modeling and feature extraction to identify exposures in blinded samples:

To estimate the level of confidence in the methodology, we used our microarray data from the NHP study as a training set. The NHP samples from 12 control and 5 VEE challenged NHP were blinded as "test" datasets.
We applied principal component analysis to examine the behavior of these samples and plotted the PCA components (Figure 8).

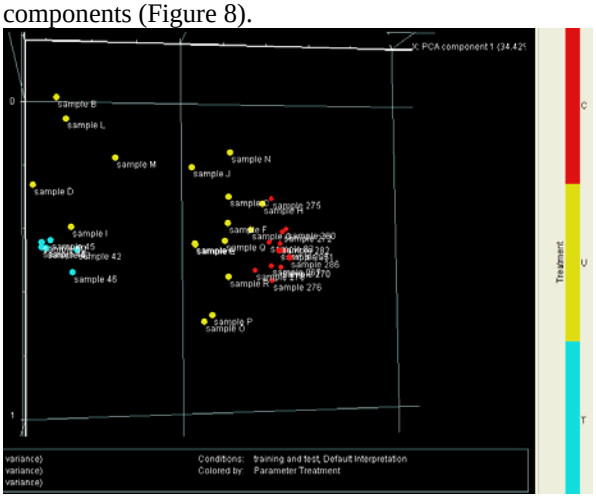

FIGURE 8. Principal component analysis of known and blinded sets. The blinded set is shown in yellow, the VEE treated samples are shown in blue and the control untreated samples were shown in red.

We used the k-nearest neighbors algorithm with a $p<0.02$. We identified 50 genes that can be predictors of exposure to VEE.

All of the 17 samples were correctly categorized as to the nature of the exposure. Using both types of training/test methods, one control NHP was categorized correctly as a "control" but had at least some indicators of common viral exposure.

CONCLUSION

Using systems biology approaches we are integrating multiple data types including clinical findings, various -omic approaches, information relating to individual/ multiple tissues affected from the onset of the exposures to develop a universal picture of host-pathogen interactions. These approaches have set the stage to begin to identify biomarkers of disease progression, host pathogen interactions and associate with appropriate therapeutic strategies as the illness proceeds.

Mapping the regulatory networks involved in the progression of infection or disease offers important insights that have the potential to lead to a broad understanding of immune modulation and shift of paradigm from the standard "one-bug-one drug" to overarching stimulation/inhibition of critical host responses.

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